Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for the Evaluation of Tuberculin Purified Protein Derivative (PPD)

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1. Introduction

1.1 Background

This is a Supplemental Assay Method (SAM) procedure for the evaluation of production lots of tuberculin purified protein derivative (PPD) in accordance with the Code of Federal Regulations, Title 9 (9 CFR), Part 113.409.

1.2 Keywords

Tuberculin; PPD; purified protein derivative; 9 CFR, Part 113.409; guinea pig; *Mycobacterium bovis; Mycobacterium avium*

2. Materials

2.1 Reagents/supplies

2.1.1 Reference PPD tuberculin, current lot. This reference is obtained from the United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Veterinary Services (VS), Center for Veterinary Biologics-Laboratory (CVB-L).

2.1.2 *Mycobacterium bovis* sensitizing agent, current lot. This reagent is available from the CVB-L.

2.1.3 *M. avium* sensitizing agent, current lot. This reagent is available from the CVB-L.

2.1.4 Phosphate buffer, PPB II

2.1.5 Metric ruler made of clear plastic

2.1.6 Needles, 20 ga x 1 in and 26 ga x 3/8 in

2.1.7 Disposable syringes, 1 ml and 3 ml

2.1.8 Pipettes, 5 ml and 25 ml

2.1.9 Glass serum bottles, 20 ml and 125 ml

2.1.10 Rubber seals and metal caps for serum bottles

2.1.11 Crimper for aluminum seals

2.1.12 Depilatory cream, Nair™ or equivalent

2.1.13 Animal clippers, equipped with #40 blade, or equivalent

2.2 Animals

Guinea pigs, 500-700 g, white-haired, nonpregnant females. Twenty-three guinea pigs are required for each lot to be tested. For each group of unknowns to be tested simultaneously, 20 guinea pigs are required for the reference PPD. All guinea pigs used for a test must be from the same source and housed and fed in the same manner.

3. Preparation for the test

3.1 Personnel qualifications/training

Technical personnel must have working knowledge of the use of general laboratory chemicals, equipment, and glassware and have specific training and experience in the safe handling of laboratory animals. They must have experience in the performance of this assay.

3.2 Selection and handling of test animals

3.2.1 Select guinea pigs that are healthy and free of external parasites and have an unblemished hair coat.

3.2.2 Examine guinea pigs the day they are received, and house according to the current version of the National Veterinary Services Laboratories (NVSL)/CVB-L Animal Users' Manual.

3.2.3 When the test is concluded, instruct the animal caretakers to euthanize and incinerate the guinea pigs and to sanitize the cages according to the current version of ARSSOP0004.

3.3 Preparation of reagents

3.3.1 Phosphate buffer PPB II--NVSL Media #30059

Na_2HPO_4	1.89 g
KH_2PO_4	0.36 g
NaCl	8 g
Distilled water	900 ml

Adjust pH to 7.2 ! 0.1. Autoclave at 121°C for 20 min. When cool, add:

5% phenol (in water)	100 ml

3.4 Preparation of supplies

3.4.1 Sterilize all glassware before use.

3.4.2 Use only sterile supplies (syringes, needles, rubber seals, metal caps, etc.).

3.5 Test animal sensitization

3.5.1 Sensitize 20 guinea pigs per lot of PPD to be evaluated. Sensitize 10 of the animals with *M. bovis* sensitinogen, and sensitize the remaining 10 animals with *M. avium* sensitinogen. For each group of PPD lots that will be tested simultaneously, similarly sensitize 20 additional guinea pigs to be used for testing the reference PPD. Wait **35 days** before performing the potency portion of the assay.

3.5.2 Administer 0.5 ml of the respective sensitinogen intramuscularly to each guinea pig. Split the dose, administering 0.25 ml into each rear leg. Use 3-ml syringes fitted with 20-ga x 1-in needles.

3.5.3 Retain 3 guinea pigs as nonsensitized controls for each unknown lot of PPD to be tested. Unsensitized controls are not necessary for the reference PPD.

4. Performance of the potency test

4.1 Preparation of guinea pigs for the potency assay

Clip the entire abdomen of each guinea pig with animal clippers. Generously apply a depilatory cream to the clipped abdomen. Wait at least 10 min. Wash off the depilatory cream with warm water and dry the abdomen with a soft towel. Allow the guinea pigs to rest for at least 4 hr before administering the tuberculin injections.

4.2 Preparation of tuberculin dilutions

4.2.1 Preparation of assay dilutions

4.2.1.1 Make 4 dilutions of the test lot of PPD, using sterile PPB II as the diluent. Dilute to achieve final concentrations of 0.6, 1.2, 2.4, and 4.8 μg protein per 0.1-ml dose, respectively. Place each dilution in a serum bottle. Cap and label the bottles.

4.2.1.2 Repeat **Section 4.2.1.1** with each additional lot of PPD to be tested and with the reference PPD.

4.3 Intradermal injection of test animals

4.3.1 Identify 4 injection sites on the abdomen of each guinea pig, with 2 sites on each side, equidistant from the midline. Sites must be spaced sufficiently far apart to avoid overlapping of subsequent skin reactions. Do not mark the sites on the abdomen with ink. Randomly assign and record which dilution (0.6, 1.2, 2.4, or 4.8 μg protein per 0.1-ml dose) will be injected into each site, or use worksheet, BBFRM0002 (current version), which contains randomly generated site assignments for each guinea pig.

4.3.2 Inject each sensitized guinea pig at the 4 defined injection sites. Inject individual guinea pigs with only a single lot of PPD, 1 site per dilution, as prepared in **Section 4.2.1**.

4.3.2.1 Administer each preparation intradermally. Use 1-ml tuberculin syringes fitted with 26-ga x 3/8-in needles.

4.3.2.2 For each lot of PPD to be tested, inject 10 guinea pigs sensitized with *M. bovis* and 10 guinea pigs sensitized with *M. avium*. Similarly inject 3 nonsensitized guinea pigs.

4.3.2.3 Inject the reference PPD into each of 10 guinea pigs sensitized with *M. bovis* and 10 guinea pigs sensitized with *M. avium*.

5. Interpretation of the test results

5.1 Recording of test results

5.1.1 Measure the test reactions at 23-25 hr following injection.

5.1.2 Measure the greater and lesser diameters of erythema and/or swelling to the closest mm at each injection site (see **Figure 1**). Gently palpate the lesion to determine the margin of the swelling, which may or may not extend beyond the margin of erythema. Record the results on the test record, BBFRM0002, current version.

5.1.3 Calculate the area of erythema and/or swelling (in mm^2) by multiplying the greater and lesser diameter measurements.

5.1.4 Determine the total area of erythema and/or swelling for each guinea pig by adding the areas of the 4 injection sites.

5.1.5 Add together the totals from **Section 5.1.4** for all guinea pigs with the same sensitization and same PPD tuberculin injection. Then divide by the number of guinea pigs in that treatment group to determine the average response per guinea pig to the given PPD tuberculin for the given type of sensitization.

Figure 1.

5.1.6 Calculate the specificity index of each lot of PPD tuberculin by subtracting the average response obtained on *M. avium*-sensitized guinea pigs injected with that lot from the average response of *M. bovis*-sensitized guinea pigs injected with that lot. Record all calculations on the test record.

5.2 Criteria for a valid test

5.2.1 The specificity index of the reference PPD must be at least 400 mm^2 for a valid test.

5.2.2 The test lot of PPD tuberculin is unsatisfactory if erythema and/or swelling is noted at the injection site(s) of 1 or more of the 3 unsensitized guinea pigs injected with that lot.

5.2.3 If the test is valid and no reactions are observed in unsensitized guinea pigs, each lot of PPD tuberculin is classified according to the following table:

Specificity Index	Classification
440 mm^2 or greater	Satisfactory
360-440 mm^2	Inconclusive
Less than 360 mm^2	Unsatisfactory

5.2.4 If a lot of PPD tuberculin is inconclusive, perform a second-stage test. If a second-stage test is not performed, the lot is unsatisfactory.

5.2.4.1 Conduct the second-stage test in a manner identical to the first, except omit the unsensitized guinea pigs.

5.2.4.2 Combine the results obtained on all guinea pigs in stages 1 and 2. Calculate the average response on the 20 guinea pigs sensitized with each antigen and calculate the specificity index.

5.2.4.3 If the cumulative specificity index is μ400 mm^2, the lot of PPD tuberculin is satisfactory; if it is <400 mm^2, the lot is unsatisfactory.

6. Reporting of test results

Report test results as described by BBSOP0020, current version.

7. References

Code of Federal Regulations, Title 9, Part 113.409, U.S. Government Printing Office, Washington, DC, 1999.